

Prenatal Sex Ratios and Expression of Sexually Dimorphic Traits in Three Snake Species

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ABSTRACT Variation in intrauterine exposure to hormones associated with variation in the sex of litter mates has well-established and far-reaching effects on sexual development in some mammals. Research on this phenomenon in reptiles is scant, but suggests that lizards may follow the mammalian model whereas snakes may be affected differently. We examined sex-specific expression of four sexually dimorphic traits (tail length, head length, ventral scale count, swimming speed) in three species of snakes (*Nerodia sipedon*, *Thamnophis sirtalis*, *T. sauritus*) relative to litter sex ratios. We found little evidence that traits in either sex were masculinized or feminized in response to variation in litter sex ratio. The one significant result appeared best explained as a statistical artifact attributable to a single litter. Our results indicate that snakes are different from the one lizard studied to date. Unlike previous suggestions that prenatal hormonal mechanisms operate differently in snakes and lizards, however, the difference appears to be that development of sexually dimorphic traits in lizards is affected by litter sex ratios whereas in snakes it is not. *J. Exp. Zool.* 305A:603–609, 2006. © 2006 Wiley-Liss, Inc.

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Prenatal exposure to hormones affects the development and expression of an array of anatomical, physiological and behavioral sexual traits in mammals (Vandenbergh, 2003). In addition to being exposed to maternally produced hormones, embryos also produce hormones that affect both their own development and that of their womb mates. Male fetuses produce more testosterone early in development and female fetuses produce more estradiol later (Ryan and Vandenbergh, 2002). Diffusion of these hormones through the amniotic fluid or via the maternal bloodstream results in fetuses affecting each other's development. Positional effects and diffusion patterns vary among species but in general, female fetuses from male-biased litters tend to be masculinized whereas males from female-biased litters tend to be feminized (Clark and Galef, '95; Ryan and Vandenbergh, 2002). Documented effects of sib-mediated intrauterine hormone exposure include changes in aggressive behavior, sexual attractiveness, duration of reproductive life and offspring sex ratio (Clark and Galef, '95).

The range of factors affected by intrauterine hormone exposure makes the phenomenon potentially relevant to multiple disciplines (e.g., population biology, animal science, medicine, psychology). How great that relevance proves to be will depend in part on how widespread the phenomenon is across different taxa. To date, almost all research on this topic has been on mammals, with most of that focused on just several species of rodents (Clark and Galef, '95; Ryan and Vandenbergh, 2002). Studies with reptiles, however, have produced results that suggest that intrauterine hormone effects may be important in viviparous members of this taxonomic group (Osypka and Arnold, 2000; Uller and Olsson, 2003; Uller et al., 2004, 2005), particularly given pronounced devel-

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opmental plasticity exhibited by reptilian embryos in response to factors such as temperature variation (e.g., Flores et al., '94). Here, we investigate intrauterine effects of litter sex ratios in three species of snakes to assess the generality of results previously reported for reptiles.

In studies of reptiles to date, intrauterine effects on sexual dimorphism were assessed by examining expression of sexually dimorphic morphological traits in offspring relative to the sex ratios of the litters from which they came. Male garter snakes (*Thamnophis*) have more tail vertebrae than females, reflected externally by more sub-caudal scales (Arnold, '88). Opposite to the prediction that females from male-biased litters should have more sub-caudal scales than their counterparts from female-biased litters, however, female *T. elegans* were more feminized in male-biased litters (Osypka and Arnold, 2000). This result could indicate that sexual differentiation occurs differently in snakes than in mammals (Osypka and Arnold, 2000). Alternatively, because sex-ratio effects were observed only in females and only in one of two populations studied, the pattern could have been a statistical artifact (Osypka and Arnold, 2000). Evidence from a viviparous lizard (*Lacerta vivipera*) did not support the hypothesis that sexual differentiation is different in reptiles than in mammals. Both sexes were more feminized (smaller heads and more ventral scales) in female-biased litters and more masculinized in male-biased litters (Uller and Olsson, 2003; Uller et al., 2005). Thus, differences in how sex ratios affect development may occur within reptiles (i.e., between snakes and lizards) rather than between reptiles and mammals.

As a step toward determining whether litter sex ratios generally affect sexual development in reptiles and whether those patterns differ between snakes and lizards, we investigated litter sex-ratio effects in three species of snakes. Two of the species were in the same genus (*Thamnophis*) used in the previous study of this phenomenon on snakes (Osypka and Arnold, 2000). We also broadened the scope of our study by considering three sexually dimorphic morphological traits and one behavioral trait. Two of the morphological traits were similar to those previously examined in reptiles.

MATERIALS AND METHODS

The study was conducted at the Queen's University Biological Station in 1990 and from 1994 through 1999. Data were collected for various

studies, but the methods used made the data suitable for our objectives here. The three species used were northern watersnakes (*Nerodia sipedon*), eastern garter snakes (*Thamnophis sirtalis*) and northern ribbonsnakes (*Thamnophis sauritus*), all of which are viviparous colubrids that are common near the Biological Station. All three species mate soon after emerging from hibernation and give birth in mid-to-late summer. Sexually mature snakes were captured opportunistically by searching appropriate habitat (Scribner and Weatherhead, '95) in April through June. Females captured soon after emergence were initially housed with males to investigate mating behavior (e.g., Kissner et al., 2005), whereas females captured later in the season were assumed to have already mated. Once the mating season ended, all males were released at their point of capture.

Females were individually marked using passive integrated transponders injected sub-cutaneously and housed either in outdoor enclosures or indoors in fiberglass tanks. Outdoor enclosures ranged in size from 5 × 5 m to 5 × 10 m. Enclosures were constructed of sheet metal 75 cm high painted to reduce reflectance, with perimeter electric fencing to discourage entry by mammals and flagging tape tied to overhead monofilament lines to discourage birds. Enclosures contained natural vegetation and cover (logs, leaf litter, concrete blocks) that provided snakes access to direct sun or shade, and either a water bowl (*T. sirtalis*) or small pond (*T. sauritus* and *N. sipedon*). Indoor tanks (74 × 59 × 55 cm³) had carpeting or wood shavings and a water bowl large enough for snakes to submerge themselves. Temperature in the tank room fluctuated with ambient temperatures but was always maintained above 20°C and tanks were provided with heat sources. In both housing conditions, snakes were fed minnows (both live and previously frozen) at least twice a week.

Late in gestation all females were housed indoors, either alone or with one other female until parturition (females housed together never gave birth simultaneously). At parturition we collected all neonates. Within 24 hr of birth we measured snout-vent length (SVL), tail length and head length and counted the ventral scales of each neonate. All measurements were done twice and the mean used for analysis. Sex was determined both by probing the cloaca for hemipenes and by hemipene eversion. Rare instances of disagreement between the two methods were

always resolved by repeating the sexing. Swimming speed was measured by chasing each neonate the length of a trough of water 2 m long and 14 cm wide with the water kept at 25°C. Each individual was tested twice and the faster of the two trials recorded as its swimming speed. Following swimming trials, we released most neonates and their mothers at the location the mother had been captured. Some neonatal *N. sipedon* were kept for further research (Kissner and Weatherhead, 2005). Complete data were not collected for every litter, so sample sizes vary among analyses. All methods were approved by the Carleton University Animal Care Committee.

Litter sex ratio was determined as the proportion of males in the litter. When litters included fully developed stillborn young, the sex of those young one was used in the calculation of the litter sex ratio and the morphological measurements were used in the analysis of sexual dimorphism. No litters included in analyses had fewer than two individuals of each sex. We first determined the extent to which traits varied with body size using linear regression of each trait on SVL for each species. Where $R^2 > 0.15$, we controlled for SVL by using relative values (calculated by dividing each measurement by SVL) in subsequent analyses. We considered this a conservative threshold below which SVL would not have a confounding effect. We determined the degree of sexual dimorphism in each of the four traits for each species using paired *t*-tests to compare mean male and female values for each litter. To test the hypothesis that litter sex ratio affected the development of sexually dimorphic traits, we used linear regression of the mean trait value within litters on litter sex ratio separately for males and females.

RESULTS

We obtained data from 103 litters of *N. sipedon*, 50 litters of *T. sirtalis* and 31 litters of *T. sauritus* (Table 1). Litter sizes and sex ratios varied

substantially among females within species, but overall mean sex ratios were remarkably close to equal for each of them (Table 1).

All four of the traits we examined varied significantly with body size (SVL) in each species, although statistical significance was a consequence of large samples more than strong SVL effects in most instances (Table 2). There were only four cases (tail length of each species and head length of *N. sipedon*) that met the criterion of SVL explaining > 15% of the variance (Table 2). In subsequent analyses, we use relative values (expressed as a proportion of SVL) for these four cases.

Overall sizes of males and females within species were not significantly different, but most other traits were sexually dimorphic (Table 3). In all three species, males had longer tails than females and tail length was the most dimorphic trait in each species, a common pattern in snakes (King, '89). Females had bigger heads than males in each species, although the difference was not significant for *T. sauritus* (Table 3). Female *N. sipedon* had more ventral scales than males while the opposite pattern occurred in both *Thamnophis* species, reflecting trends in SVL differences (Table 3). Females swam faster than males, although not significantly so in *N. sipedon* (Table 3).

There was little evidence that expression of sexually dimorphic traits was affected by litter sex ratios. Tail length was the most dimorphic trait

TABLE 2. Variance explained (R^2) by body length in each of the variables used in sexual size dimorphism analyses determined by linear regression of each variable on SVL¹

| | Tail length | Head length | Ventral scales | Swim speed |
|--------------------|-------------|-------------|----------------|------------|
| <i>N. sipedon</i> | 0.34 | 0.48 | 0.01 | 0.09 |
| <i>T. sirtalis</i> | 0.46 | 0.15 | 0.15 | 0.03 |
| <i>T. sauritus</i> | 0.60 | 0.07 | 0.04 | 0.08 |

¹Each analysis was run using values for individual neonates of both sexes for each species (all $P < 0.001$).

TABLE 1. Sample sizes and sex ratios for *N. sipedon*, *T. sirtalis* and *T. sauritus* litters

| | Number of broods | Total neonates | Litter size | | Proportion male | |
|--------------------|------------------|----------------|-------------|-------|-----------------|-----------|
| | | | Mean | Range | Mean | Range |
| <i>N. sipedon</i> | 103 | 1,852 | 18.0 | 7–37 | 0.490 | 0.13–0.75 |
| <i>T. sirtalis</i> | 50 | 686 | 13.7 | 7–28 | 0.502 | 0.20–0.77 |
| <i>T. sauritus</i> | 31 | 312 | 10.1 | 5–21 | 0.500 | 0.18–0.80 |

TABLE 3. Mean values by sex and sexual dimorphism in overall size (SVL) and in each of the individual variables¹

| | | Male mean | Female mean | <i>t</i> | <i>P</i> | df |
|--------------------|----------------------|-----------|-------------|----------|----------|-----|
| <i>N. sipedon</i> | SVL | 16.78 | 16.83 | -1.64 | 0.10 | 102 |
| | Relative tail length | 0.34 | 0.29 | 60.65 | <0.0001 | 102 |
| | Relative head length | 1.28 | 1.31 | -17.46 | <0.0001 | 70 |
| | Ventral scales | 143.6 | 144.2 | -4.64 | <0.0001 | 87 |
| | Swim speed | 3.62 | 3.69 | -0.59 | 0.56 | 48 |
| <i>T. sirtalis</i> | SVL | 14.57 | 14.51 | 1.36 | 0.18 | 49 |
| | Relative tail length | 0.31 | 0.28 | 28.20 | <0.0001 | 49 |
| | Head length | 9.31 | 9.52 | -10.48 | <0.0001 | 48 |
| | Ventral scales | 149.6 | 146.1 | 19.02 | <0.0001 | 49 |
| | Swim speed | 5.66 | 6.38 | -4.32 | <0.0001 | 42 |
| <i>T. sauritus</i> | SVL | 14.85 | 14.77 | 1.03 | 0.31 | 30 |
| | Relative tail length | 0.42 | 0.41 | 7.44 | <0.0001 | 30 |
| | Head length | 9.22 | 9.26 | -1.26 | 0.22 | 30 |
| | Ventral scales | 157.1 | 155.6 | 5.81 | <0.0001 | 28 |
| | Swim speed | 6.39 | 7.18 | -3.12 | 0.005 | 24 |

¹Sexual dimorphism determined using paired *t*-tests of litter means for males and females. Relative values (corrected for SVL) used where SVL explained >15% of the variance in the trait. Length measured in mm; swim speed measured in sec.

and there was no evidence that tails became longer in males or females in response to more male-biased sex ratios (Fig. 1). Of 20 analyses of sexually dimorphic traits, only two produced significant relationships with litter sex ratio (Table 4). Mean head length of both male and female *T. sirtalis* declined as litter sex ratios become more male biased. Given that female *T. sirtalis* have larger heads than males, this pattern is consistent with the hypothesis that both sexes become more masculinized as litters become more male biased. However, the pattern appears to be the result of one or two female-biased litters in which neonates had particularly large heads (Fig. 2). Excluding just the litter with the largest mean head length from the analysis causes the pattern to become non-significant ($r = -0.16$ and -0.19 , $P = 0.29$ and 0.20 , for males and females, respectively).

DISCUSSION

There was little evidence that development of sexually dimorphic traits was affected by litter sex ratios. Across the three snake species, tail length was the most sexually dimorphic trait but did not vary with litter sex ratio in either males or females. The only result suggestive of a litter sex-ratio effect (head length in *T. sirtalis*) was best interpreted as a statistical artifact attributable to one litter with extreme values. A potential explanation for our failure to find sex-ratio effects was that sexual dimorphism was quite limited for

most traits. Given that available sample sizes were sufficient to detect sexual dimorphism in most traits, however, sample sizes should also have been sufficient to detect sex-ratio effects had they occurred.

Osyпка and Arnold (2000) found apparent evidence that sub-caudal scale counts in *T. elegans* were affected by litter sex ratio, albeit opposite the pattern predicted from mammalian studies (females were feminized in male-biased litters). Although this result could indicate that reptiles have a different mechanism of sexual development than mammals, Osyпка and Arnold (2000) also proposed the alternative interpretation that their result could have been a statistical artifact because the pattern only occurred in females and only in one of two populations studied. Our failure to find evidence that tail lengths were affected by litter sex ratios in three snake species, two of which are congeners of *T. elegans*, supports the statistical artifact interpretation of Osyпка and Arnold's (2000) results.

Two studies of a lizard species did find that litter sex ratio affected neonatal head length and ventral scale count, both sexually dimorphic traits (Uller and Olsson, 2003; Uller et al., 2005). Although the results for head length were characterized as being sex-specific, affecting sons and daughters differently (Uller and Olsson, 2003), both sexes appeared to be affected similarly, consistent with mammalian patterns (Clark and Galef, '95; Ryan and Vandenberg, 2002). Both sexes were masculinized in male-biased litters and feminized in

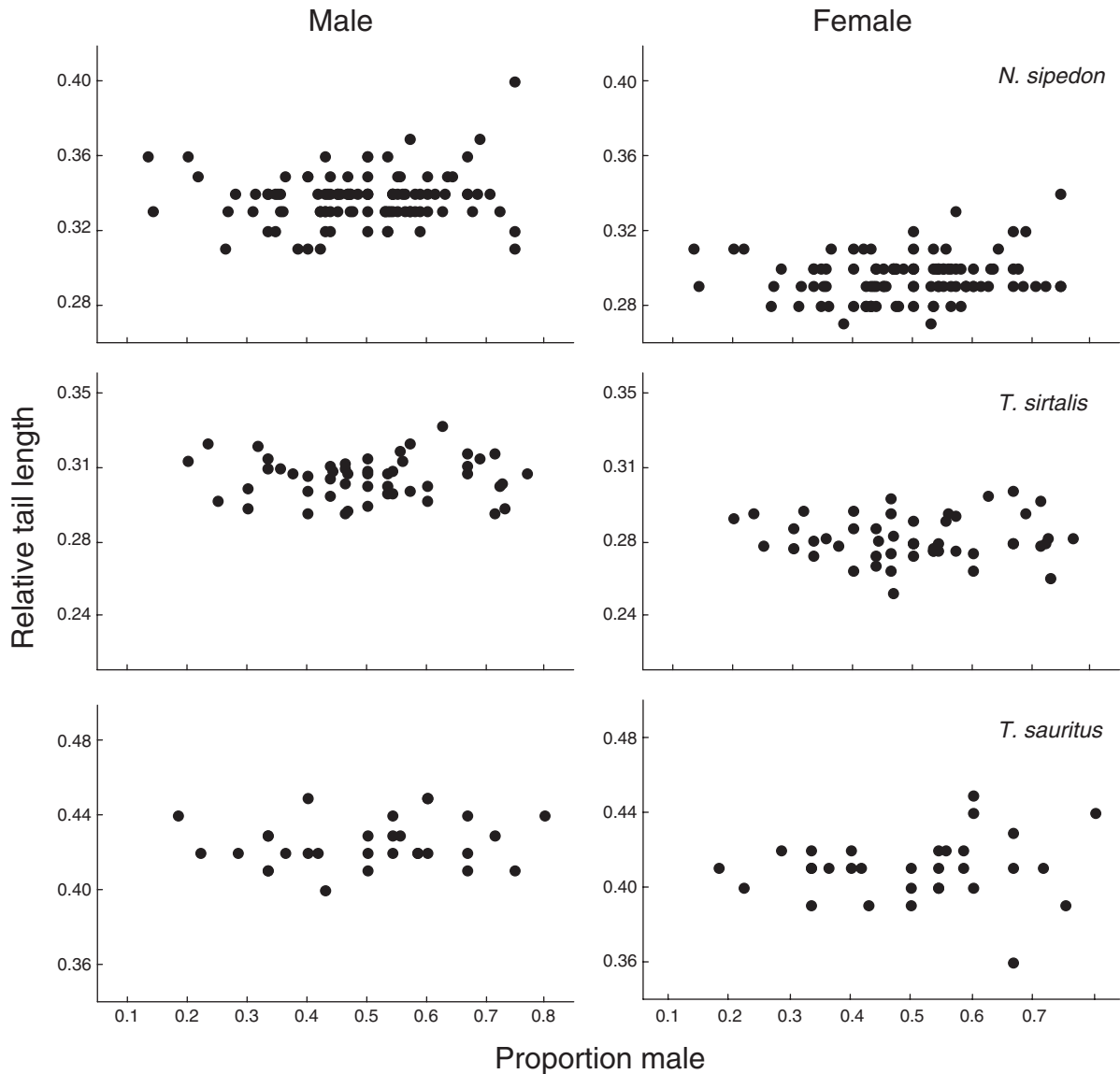


Fig. 1. Mean relative tail lengths of male and female neonates relative to litter sex ratio (proportion male).

female-biased litters relative to neonates of the same sex from litters with even sex ratios. Given that lizards (at least the one species studied thus far) and mammals exhibit similar effects of prenatal sex ratios on development of sexually dimorphic traits, why does this not occur in snakes? Sexual development is hormonally mediated in snakes (Shine and Crews, '88), but diffusion of hormones between developing offspring might be more constrained in snakes than in lizards or mammals.

Additional research is required to determine the generality of the snake-lizard difference in pre-

natal sex-ratio effects and the underlying physiological mechanisms. More broadly, presence of these effects in some taxa but not others raises interesting evolutionary questions. If intrauterine sex-ratio effects reduce mean offspring fitness, selection should favor reduction of those effects in species that exhibit them. For example, adaptive sex ratio adjustment might be constrained (Uller, 2003). Alternatively, because sex-ratio effects alter offspring traits that are important later in life (Clark and Galef, '95), they may provide mothers a mechanism for varying offspring traits adaptively via litter sex allocation, which should favor

TABLE 4. Relationship between sexually dimorphic traits and litter sex ratios determined by linear regression¹

| | Trait | Sex | <i>r</i> | <i>F</i> | <i>P</i> | df |
|--------------------|----------------------|--------|----------|----------|----------|-----|
| <i>N. sipedon</i> | Relative tail length | Male | 0.08 | 0.72 | 0.40 | 102 |
| | | Female | 0.10 | 1.11 | 0.29 | |
| | Relative head length | Male | -0.04 | 0.09 | 0.77 | 70 |
| | | Female | -0.05 | 0.16 | 0.69 | |
| | Ventral scales | Male | -0.01 | 0.01 | 0.91 | 87 |
| | | Female | -0.02 | 0.05 | 0.82 | |
| <i>T. sirtalis</i> | Relative tail length | Male | -0.09 | 0.40 | 0.53 | 49 |
| | | Female | -0.07 | 0.23 | 0.64 | |
| | Head length | Male | -0.30 | 4.72 | 0.03 | 48 |
| | | Female | -0.28 | 3.95 | 0.05 | |
| | Ventral scales | Male | -0.07 | 0.27 | 0.60 | 49 |
| | | Female | -0.14 | 1.02 | 0.32 | |
| Swim speed | Male | -0.04 | 0.07 | 0.79 | 42 | |
| | Female | 0.12 | 0.57 | 0.45 | | |
| <i>T. sauritus</i> | Relative tail length | Male | 0.11 | 0.37 | 0.55 | 30 |
| | | Female | 0.12 | 0.39 | 0.54 | |
| | Ventral scales | Male | 0.04 | 0.04 | 0.85 | 28 |
| | | Female | -0.01 | 0.003 | 0.96 | |
| | Swim speed | Male | 0.23 | 1.34 | 0.26 | 24 |
| | | Female | 0.13 | 0.39 | 0.54 | |

¹Mean trait values within litters for males and females were used in the analyses. Relative values (corrected for SVL) were used when SVL explained >15% of the variance in a trait.

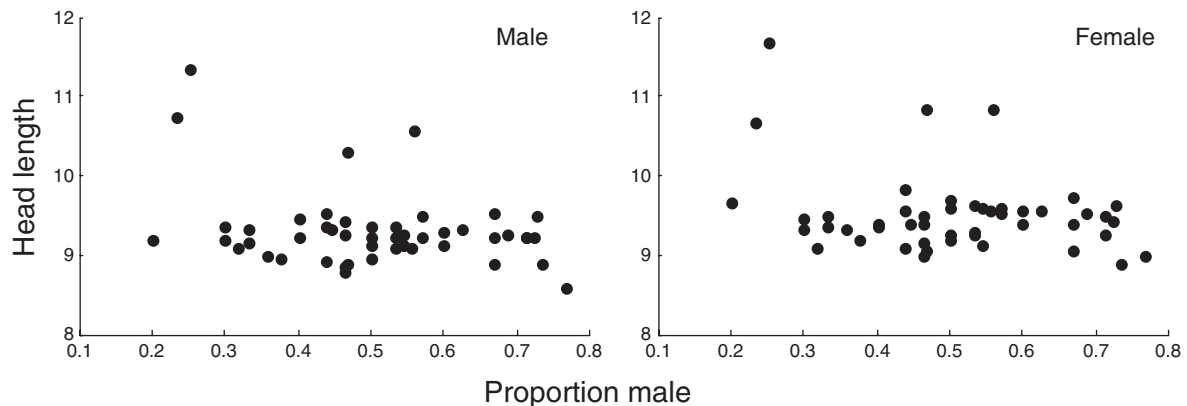


Fig. 2. Mean head lengths (mm) of male and female neonates of *T. sirtalis* relative to litter sex ratio (proportion male).

sex-ratio effects in species that lack them. Comparative studies of taxa with and without these mechanisms seem likely to be a fruitful approach for investigating these issues.

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